Association of a common vitamin D-binding protein polymorphism with inflammatory bowel disease

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Abstract: OBJECTIVE: Inflammatory bowel diseases (IBDs), Crohn's disease, and ulcerative colitis (UC), are multifactorial disorders, characterized by chronic inflammation of the intestine. A number of genetic components have been proposed to contribute to IBD pathogenesis. In this case-control study, we investigated the association between two common vitamin D-binding protein (DBP) genetic variants and IBD susceptibility. These two single nucleotide polymorphisms (SNPs) in exon 11 of the DBP gene, at codons 416 (GAT>GAG; Asp>Glu) and 420 (ACG>AAG; Thr>Lys), have been previously suggested to play roles in the etiology of other autoimmune diseases. METHODS: Using TaqMan SNP technology, we have genotyped 884 individuals (636 IBD cases and 248 non-IBD controls) for the two DBP variants. RESULTS: On statistical analysis, we observed that the DBP 420 variant Lys is less frequent in IBD cases than in non-IBD controls (allele frequencies, P=0.034; homozygous carrier genotype frequencies, P=0.006). This inverse association between the DBP 420 Lys and the disease remained significant, when non-IBD participants were compared with UC (homozygous carrier genotype frequencies, P=0.022) or Crohn's disease (homozygous carrier genotype frequencies, P=0.016) patients separately. Although the DBP position 416 alone was not found to be significantly associated with IBD, the haplotype $DBP_2, consisting of 416Asp and 420Lys, was more frequent in the non-IBD population, particularly notably when compared with the UC group (Odds ratio, 4.390). CONCLUSION: Our study adds DBP to the list of potential genes that contribute to the complex genetic etiology of IBD, and further emphasize$

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Association of a common vitamin D-binding protein (DBP) polymorphism with inflammatory bowel disease

Running Head: DBP SNP and IBD

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Abstract

Objective Inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial disorders, characterized by chronic inflammation of the intestine. A number of genetic components have been proposed to contribute to IBD pathogenesis. In this case-control study, we investigated the association between two common vitamin D-binding protein (DBP) genetic variants and IBD susceptibility. These two single nucleotide polymorphisms in exon 11 of the *DBP* gene, at codons 416 (*GAT*>*GAG*; Asp>Glu) and 420 (*ACG*>*AAG*; Thr>Lys), have previously been suggested to play roles in the etiology of other autoimmune diseases.

Methods Using TaqMan SNP technology, we have genotyped 884 individuals (636 IBD cases and 248 non-IBD controls) for the two DBP variants.

Results Upon statistical analysis, we observed that the DBP 420 variant Lys is less frequent in IBD cases than in non-IBD controls (allele frequencies, p=0.034; homozygous carrier genotype frequencies, p=0.006). This inverse association between the DBP 420 Lys and the disease remained significant, when non-IBD subjects were compared with UC (homozygous carrier genotype frequencies, p=0.022) or CD (homozygous carrier genotype frequencies, p=0.016) patients separately. While the DBP position 416 alone was not found to be significantly associated with IBD, the haplotype *DBP_2*, consisting of 416 Asp and 420 Lys, was more frequent in the non-IBD population, particularly notably when compared to the UC group (OR 4.390).

Conclusion Our study adds DBP to the list of potential genes that contribute to the complex genetic etiology of IBD, and further emphasizes the association between vitamin D homeostasis and intestinal inflammation.

Key words:
Inflammatory bowel disease, vitamin D-binding protein, Crohn's disease, ulcerative colitis, vitamin D homeostasis

Introduction

The biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 (vitamin D_3) controls the serum calcium concentration and overall calcium homeostasis, and low vitamin D_3 levels result in acceleration of bone turnover. Furthermore, vitamin D_3 contributes to immunomodulation and possesses antiproliferative properties [1]. In the intestine, vitamin D_3 has additional important functions, such as promotion of the integrity of mucosal tight junctions [2], enhancement of epithelial folate absorption [3], and activation of intestinal cytochrome P450 3A4 expression [4].

Vitamin D-binding protein (DBP) is a multifunctional glycosylated protein, encoded by the *GC* (gc-globulin = group-specific component) gene on chromosome 4 in humans [5-9, and references therein]. It belongs to the albumin multigene cluster family of proteins, together with α-fetoprotein and albumin itself. DBP is synthesized predominantly in liver parenchymal cells, as a precursor form of 474 amino acids, which is processed to the mature form of 458 amino acids (~58 kDa). The primary structure of DBP contains 28 cysteine residues, which can form multiple disulfide bonds. DBP primarily locates in circulating plasma at the concentration of ~5 μM, but can also be found in ascitic and cerebrospinal fluids, and in cytoplasm of nucleated cells, as well as surface membranes of B and T lymphocytes. In plasma, DBP mediates vitamin D metabolite transport and fatty acid transport to target tissues. Additional reported functions of DBP include inhibition of actin polymerization and macrophage activation.

The DBP gene in humans exists as three common variants, and over 100 rare variants [9]. The three major DBP isoforms are referred to as Gc1F (wild-type), Gc1S, and Gc2. All three isoforms exhibit comparable chemotactic activity [10], but the total levels of DBP in circulation appear to be determined by the Gc phenotype [11]. The isotypes are distinguished by distinct combinations of substitutions at amino acid positions 416

(Asp>Glu) and 420 (Lys>Thr). Relative to the wild-type Gc1F protein, the Gc1s is defined by containing the Asp416Glu polymorphism, and the Gc2 contains the Thr420Lys polymorphism. Enzymatic processing of the DBP O-linked carbohydrate to GalNac results in the transformation of the DBP molecules into the macrophage-activating factors (MAF). MAF is a lymphokine that primes macrophages, when required during the host defence responses in infectious and inflammatory diseases and against tumours [12]. Furthermore, MAF can induce macrophage cell death by enhancing caspase activity at sites of infection or inflammation [13].

Several studies have proposed a link between genetic variants of DBP and disease in humans. These include many conditions of suspected autoimmune etiology, such as Graves' disease, diabetes, and osteoporosis [reviewed in 9]. Inflammatory bowel disease (IBD) is a condition characterized by recurrent inflammation of the intestinal mucosa, and is caused by deranged function of mucosal immune system in genetically susceptible subjects [reviewed in 14]. IBD etiology clearly involves a complex interaction of genetic, environmental, and immunomodulatory factors. Two major forms of IBD exist. In Crohn's disease (CD), the entire gastrointestinal tract may be affected, although the most frequent site of inflammation is the ileum, whereas in ulcerative colitis (UC) the inflammation typically affects the colon. In CD pathogenesis, a strong genetic component has been suggested by the concordance of 63.6% in monozygotic twins, but only of 3.6% in dizygotic ones. In UC, the concordance of monozygotic twins is lower (6%), implying that genetic factors may play a somewhat smaller role in this disease [15]. IBD is more prevalent in northern Europe and North America, where less vitamin D is synthesized in the skin upon lower exposure to sunlight [16]. Vitamin D deficiency also frequently occurs in IBD patients, even when the disease itself may be in a state of remission [17].

Genetic variants of the nuclear receptor for vitamin D_3, VDR, the chief executor of vitamin D_3 actions within cell nuclei, have previously been suggested to be associated with inflammatory bowel disease (IBD) [18,19]. Here, we have studied whether two common variants of the other important player in vitamin D homeostasis, DBP, are associated with IBD and its subtypes UC and CD.

Materials and methods

Study subjects

The study population was Swiss, and comprised of 248 healthy subjects and 636 IBD patients, from which 232 were diagnosed to suffer from UC and 404 from CD. The IBD subjects were recruited at the centers participating in the Swiss Inflammatory Bowel Disease Cohort Study (SIBDCS) [20]. For the IBD patients, the diagnosis of UC or CD was confirmed by the study investigators based on clinical presentation, endoscopic findings, and histology. Non-IBD controls were recruited from gastroenterological patients undergoing surveillance colonoscopy. History of colorectal cancer was employed as an exclusion criteria for both IBD patients and non-IBD control subjects.

DNA extraction

Genomic DNAs were extracted from either EDTA-blood or intestinal biopsies using the QIAamp DNA Mini Kit (QIAGEN, Hombrechtikon, Switzerland) or the TRIzol reagent (Invitrogen, Basel, Switzerland), respectively, according to the manufacturer's instructions.

Genotyping of *DBP* single nucleotide polymorphisms

Genotyping of the two *DBP* SNPs was performed using TaqMan allelic discrimination assays. The cycling was performed on an 7900HT Fast Real-Time PCR system (Applied Biosystems, Rotkreuz, Switzerland) by using custom-made TaqMan SNP Genotyping Assays. The TaqMan primer and probe sequences were as follows: The Asp416Glu variant: Left primer, TGGCAGAGCGACTAAAAGCA; right primer, CTTGTTAACCAGCTTTGCCAGTTC; VIC-labelled probe AAATTGCCTGATGCCAC; FAM-labelled probe TTGCCTGAGGCCAC. The Thr420Lys variant: Left primer, CGACTAAAAGCAAAATTGCCTGATG; right primer CTGAGTGCTTGTTAACCAGCTTTG; VIC-labelled probe CACCCACGGAACTG; FAM-labelled probe CACCCAAGGAACTG. All

fluorescent probes had a non-fluorescent quencher at 3'-end. The genomic DNAs were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and diluted to a final concentration of 10 ng/µl. The amplification run conditions were: Once 50°C for 2 min, once 95°C for 10 min, 45 times 95°C for 15 sec and 60°C for 1 min.

Ethical considerations

All subjects provided their written informed consent to be included in the study. Ethical approvals were obtained from the local medical ethical committees of all study sites involved in the Swiss IBD Cohort Study.

Statistical analysis

Statistical analysis was performed using the software packages SPSS 18 (SPSS Inc., Chicago, IL) and GraphPad Prism (GraphPad Software Inc., San Diego, CA). The Chi-square test or Fisher's exact test were used to determine associations between individual SNPs and subject phenotypes. The PHASE software was used to calculate the haplotypes in the *DBP* gene on the basis of a Bayesian interference algorithm [21]. Linkage disequilibrium (LD) was calculated using the r^2 statistics. Calculations were performed using the software package Haploview (www.haploview.com).

Results

Study population

The study population was recruited at centers participating in the Swiss IBD Cohort study (SIBDCS). Detailed demographic data of the entire cohort consisting of 884 subjects is shown in Table 1.

DBP sequence variability

The two *DBP* variants selected for the study are single nucleotide substitutions. The genotype frequencies in all groups were in Hardy-Weinberg equilibrium. The obtained allele and genotype frequencies are given in Table 2.

Genetic variation in the *DBP* gene and IBD

The DBP variant 416 Glu was not found to be significantly associated with the diagnosis of IBD, although there was a tendency for this variant to be more common in the IBD population than in healthy subjects (Tables 2 and 3). The DBP variant 420 Lys, on the other hand, was more common in the healthy population than in the IBD cohort. The minor allele frequency was significantly ($p=0.034$) lower in the IBD population. The level of significance became much more notable ($p=0.006$), when the numbers of carriers of the wild-type genotype were compared to the numbers of homozygous SNP carrier in non-IBD and IBD populations. Upon subgrouping the IBD patients, the differences in the frequencies of the rarer 420 Lys variant remained significant for both UC ($p=0.022$) and CD ($p=0.016$). Similarly, when adjusted to age and gender, the p values remained significant.

In addition to the two above-mentioned coding *DBP* SNPs, we also genotyped the study population for three *DBP* promoter SNPs, namely -1424, -944, and -39, previously studied in

the context of prostate cancer risk [22]. All the subjects in all subgroups of the current cohort, both IBD and non-IBD, were homozygous for the wild-type allele at these three positions (data not shown).

DBP haplotypes and IBD

All individuals, for whom genotype determination could be performed for both DBP variants under study were included in the haplotype prediction analysis. Thus, 185 non-IBD subjects, 225 UC cases and 393 CD cases were included in this analysis. The predicted frequencies for all four possible *DBP* haplotypes are shown in Table 4. The haplotype *DBP_2* containing the wild-type allele for the position 416 and the rarer allele for the position 420, was predicted to be significantly less frequent in the IBD population than in non-IBD subjects ($p=0.0005$) and this inverse association with the disease remained significant when either the UC ($p=0.0003$) or the CD ($p=0.013$) patients were compared with the non-IBD group separately (Table 5). The linkage equilibria between the variants at positions 416 and 420 of DBP were moderate (non-IBD controls $r^2=0.61$; IBD cases $r^2=0.78$).

Discussion

The etiology of IBD is complex, and a wide range of factors, both genetic and environmental, are believed to play roles in IBD pathophysiology. Variants in more than 70 genes have been identified that may potentially be associated with IBD [23,24]. Many of these encode genes that modulate immune responses and antimicrobial defence in the intestine, and are thus involved in maintaining the integrity of the intestinal wall epithelium [14,23]. Vitamin D metabolites have anti-inflammatory functions, and participate in the maintenance of tight junctions between intestinal epithelial cells [2]. While the other major player of vitamin D homeostasis, the vitamin D receptor VDR, has previously been genetically linked to IBD [18,19], we show here that a common genetic variant of DBP, the main transporter of vitamin D to its target tissues in the plasma, is significantly associated with IBD. This association is significant in both our UC and CD cohorts. The rarer Lys allele at position 420 appears to have a protective role against IBD, as it is more common in healthy subjects. The other DBP variant tested 416 Glu did not alone show a significant association with IBD, although it exhibited a tendency to be more frequent in IBD cases than control subjects, potentially thus acting as a factor contributing to the pathogenesis. The haplotype *DBP_2*, consisting of variants 416 Asp and 420 Lys, was significantly more frequent in the non-IBD population, more notably in comparison with the UC (OR 4.390).

Low 25-hydroxyvitamin D_3 levels are associated with insulin resistance and metabolic syndrome, and genetic variation in codon positions 416 and 420 has previously been proposed to be associated with diabetes [25,26], although this association remains somewhat controversial [27,28]. Furthermore, the DBP Lys allele at codon 420 has been associated with Graves' disease in a Polish population [29], although in this case the lysine was in fact more common in the disease group. This points to potentially differing roles of

DBP in the etiologies of IBD and Graves' disease, even if both represent autoimmune disorders.

The exact functional consequences of the amino acid exchanges at positions 416 and 420 of the DBP protein remain somewhat unclear. This region of the protein is located adjacent to the actin-binding domain of DBP (amino acids 350-403), but is more distant from the amino-terminal vitamin D-binding domain [30]. In the DBP form carrying the lysine in position 420, a major site for O-linked trisaccharide glycosylation is removed [31]. It will be of great interest to verify whether the efficiency of vitamin D transport by DBP is affected by this amino acid substitution, and in what manner. Given its inverse association with IBD reported here, it is perhaps paradoxical that the Gc2 isoform of DBP carrying the lysine in codon position 420 has been associated with lower plasma concentrations of vitamin D_3 metabolites than observed for the carriers of the other Gc alleles [11]. It has also been shown that the Gc2 isoform has reduced affinity to vitamin D metabolites [32]. We do not at this stage understand the exact mechanism by which a DBP isoform that exhibits lower affinity to vitamin D exerts a protective effect against IBD, as suggested by our current findings. It is conceivable that the reduced affinity could result in enhanced release of vitamin D from DBP to the intestinal target tissue that is susceptible to inflammation.

Patients suffering from IBD are frequently on long-term treatment with vitamin D_3, in addition to calcium, as a prophylaxis against osteoporosis and osteopenia [33]. It will be of interest to further explore whether the IBD-associated DBP variants are associated with altered clinical response to vitamin D_3 supplementation. In conclusion, we have for the first time shown an association between a chief component of vitamin D metabolism, DBP, and diagnosis of IBD, further supporting the importance of vitamin D homeostasis in this chronic inflammatory disease.

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Footnotes

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Table 1. Demographic data of the study population

Total population (N = 884)	
Male	404 (45.7%)
Female	480 (54.3%)
Age (Mean +/- SD)	43.0 (+/-15.7)
Age (Median)	41
Age (Minimum)	16
Age (Maximum)	82
Non-IBD (N = 248; 28.1% of total population)	
Male	93 (37.5%)
Female	155 (62.5%)
Age (Mean +/- SD)	44.2 (+/- 17.0)
Age (Median)	42
Age (Minimum)	20
Age (Maximum)	81
IBD (UC + CD) (N = 636; 71.9% of total population)	
Male	311 (48.9%)
Female	325 (51.1%)
Age (Mean +/- SD)	42.5 (+/- 15.1)
Age (Median)	41
Age (Minimum)	16
Age (Maximum)	82
UC (N = 232; 26.2% of total population)	
Male	120 (51.7%)
Female	112 (48.3%)
Age (Mean +/- SD)	45.3 (+/- 15.0)
Age (Median)	44
Age (Minimum)	18
Age (Maximum)	82
CD (N = 404; 45.7% of total population)	
Male	191 (47.3%)
Female	213 (52.7%)
Age (Mean +/- SD)	40.8 (+/- 15.0)
Age (Median)	39
Age (Minimum)	16
Age (Maximum)	79

Table 2. Determined allele and genotype frequencies for the DBP SNPs Asp416Glu and Thr420Lys

Asp416Glu

Total subjects successfully genotyped: N = 817 (92.4% of total population)

Male	372 (45.5%)
Female	445 (54.5%)
Non-IBD	198 (24.2% of total genotyped subjects)
IBD	619 (75.8% of total genotyped subjects)
UC	227 (27.8% of total genotyped subjects)
CD	392 (48.0% of total genotyped subjects)

Minor allele frequencies

Non-IBD	39.4%
IBD	42.3%
UC	44.3%
CD	41.2%

Genotype frequencies	WT hom	het	SNP hom
Non-IBD	72 (36.4%)	96 (48.5%)	30 (15.2%)
IBD	198 (32.0%)	318 (51.4%)	103 (16.6%)
UC	68 (30.0%)	117 (51.5%)	42 (18.5%)
CD	130 (33.2%)	201 (51.3%)	61 (15.6%)

Thr420Lys

Total subjects successfully genotyped: N = 827 (93.9% of total population)

Male	377 (45.6%)
Female	450 (54.4%)
Non-IBD	197 (23.8% of total genotyped subjects)
IBD	630 (76.2% of total genotyped subjects)
UC	228 (27.6% of total genotyped subjects)
CD	402 (48.6% of total genotyped subjects)

Minor allele frequencies

Non-IBD	33.0%
IBD	27.5%
UC	28.1%
CD	27.1%

Genotype frequencies	WT hom	het	SNP hom
Non-IBD	94 (47.7%)	76 (38.6%)	27 (13.7%)
IBD	330 (52.4%)	254 (40.3%)	46 (7.3%)
UC	115 (50.4%)	98 (43.0%)	15 (6.6%)
CD	215 (53.5%)	156 (38.8%)	31 (7.7%)

WT hom, wild-type homozygote; het, heterozygote; SNP hom, homozygous SNP carrier.

Table 3. Allele and genotype association analysis for the DBP SNPs Asp416Glu and Thr420Lys in IBD vs. non-IBD groups

Asp416Glu	**p**	**p'**	**OR**	**CI**
Allele frequencies:				
Non-IBD vs. IBD:	0.303		0.886	0.703-1.116
Genotype frequencies:				
Non-IBD vs. IBD:				
WT vs. SNP carrier:	0.254	0.319	1.215	0.869-1.699
WT vs. SNP hom:	0.372	0.382	1.248	0.766-2.034

Thr420Lys	**p**	**p'**	**OR**	**CI**
Allele frequencies:				
Non-IBD vs. IBD:	0.034*		1.301	1.019-1.660
Genotype frequencies:				
Non-IBD vs. IBD:				
WT vs. SNP carrier:	0.253	0.230	0.830	0.602-1.143
WT vs. SNP hom:	0.006**	0.008**	0.485	0.286-0.822
Non-IBD vs. UC:				
WT vs. SNP carrier:	0.576	0.688	0.897	0.612-1.313
WT vs. SNP hom:	0.022*	0.041*	0.454	0.228-0.903
Non-IBD vs. CD:				
WT vs. SNP carrier:	0.185	0.178	0.794	0.564-1.117
WT vs. SNP hom:	0.016*	0.017*	0.502	0.284-0.888

p', p value adjusted to age and gender; OR, odds ratio; CI, confidence interval.

Table 4. Predicted haplotype frequencies for the DBP SNPs Asp416Glu and Thr420Lys in IBD vs. non-IBD groups

Haplotype		**Haplotype frequencies**			
		non-IBD	IBD	UC	CD
DBP_1	*TC* (WT)	198 (53.5%)	683 (55.3%)	245 (54.4%)	438 (55.7%)
DBP_2	*TA*	24 (6.5%)	33 (2.7%)	7 (1.6%)	26 (3.3%)
DBP_3	*GC*	51 (13.8%)	220 (17.8%)	82 (18.2%)	138 (17.6%)
DBP_4	*GA*	97 (26.2%)	300 (24.3%)	116 (25.8%)	184 (23.4%)

The haplotype significantly associated with IBD is underlined

Table 5. Haplotype association analysis for the DBP SNPs Asp416Glu and Thr420Lys in IBD vs. non-IBD groups

Haplotype	p	OR	CI
DBP_1 TC (WT)			
Non-IBD vs. IBD	0.549	0.931	0.738-1.175
DBP_2 TA			
Non-IBD vs. IBD	0.0005***	2.529	1.475-4.336
Non-IBD vs. UC	0.0003***	4.390	1.869-10.31
Non-IBD vs. CD	0.013*	2.028	1.147-3.583
DBP_3 GC			
Non-IBD vs. IBD	0.068	0.737	0.530-1.024
DBP_4 GA			
Non-IBD vs. IBD	0.417	1.116	0.856-1.454

The haplotype significantly associated with IBD is underlined.

OR, odds ratio; CI, confidence interval.

Association of a common vitamin D-binding protein (DBP) polymorphism with inflammatory bowel disease

Running Head: DBP SNP and IBD

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Abstract

Objective Inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial disorders, characterized by chronic inflammation of the intestine. A number of genetic components have been proposed to contribute to IBD pathogenesis. In this case-control study, we investigated the association between two common vitamin D-binding protein (DBP) genetic variants and IBD susceptibility. These two single nucleotide polymorphisms in exon 11 of the *DBP* gene, at codons 416 (*GAT*>*GAG*; Asp>Glu) and 420 (*ACG*>*AAG*; Thr>Lys), have previously been suggested to play roles in the etiology of other autoimmune diseases.

Methods Using TaqMan SNP technology, we have genotyped 884 individuals (636 IBD cases and 248 non-IBD controls) for the two DBP variants.

Results Upon statistical analysis, we observed that the DBP 420 variant Lys is less frequent in IBD cases than in non-IBD controls (allele frequencies, p=0.034; homozygous carrier genotype frequencies, p=0.006). This inverse association between the DBP 420 Lys and the disease remained significant, when non-IBD subjects were compared with UC (homozygous carrier genotype frequencies, p=0.022) or CD (homozygous carrier genotype frequencies, p=0.016) patients separately. While the DBP position 416 alone was not found to be significantly associated with IBD, the haplotype *DBP_2*, consisting of 416 Asp and 420 Lys, was more frequent in the non-IBD population, particularly notably when compared to the UC group (OR 4.390).

Conclusion Our study adds DBP to the list of potential genes that contribute to the complex genetic etiology of IBD, and further emphasizes the association between vitamin D homeostasis and intestinal inflammation.

Key words:
Inflammatory bowel disease, vitamin D-binding protein, Crohn's disease, ulcerative colitis, vitamin D homeostasis

Introduction

The biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 (vitamin D_3) controls the serum calcium concentration and overall calcium homeostasis, and low vitamin D_3 levels result in acceleration of bone turnover. Furthermore, vitamin D_3 contributes to immunomodulation and possesses antiproliferative properties [1]. In the intestine, vitamin D_3 has additional important functions, such as promotion of the integrity of mucosal tight junctions [2], enhancement of epithelial folate absorption [3], and activation of intestinal cytochrome P450 3A4 expression [4].

Vitamin D-binding protein (DBP) is a multifunctional glycosylated protein, encoded by the *GC* (gc-globulin = group-specific component) gene on chromosome 4 in humans [5-9, and references therein]. It belongs to the albumin multigene cluster family of proteins, together with α-fetoprotein and albumin itself. DBP is synthesized predominantly in liver parenchymal cells, as a precursor form of 474 amino acids, which is processed to the mature form of 458 amino acids (~58 kDa). The primary structure of DBP contains 28 cysteine residues, which can form multiple disulfide bonds. DBP primarily locates in circulating plasma at the concentration of ~5 μM, but can also be found in ascitic and cerebrospinal fluids, and in cytoplasm of nucleated cells, as well as surface membranes of B and T lymphocytes. In plasma, DBP mediates vitamin D metabolite transport and fatty acid transport to target tissues. Additional reported functions of DBP include inhibition of actin polymerization and macrophage activation.

The DBP gene in humans exists as three common variants, and over 100 rare variants [9]. The three major DBP isoforms are referred to as Gc1F (wild-type), Gc1S, and Gc2. All three isoforms exhibit comparable chemotactic activity [10], but the total levels of DBP in circulation appear to be determined by the Gc phenotype [11]. The isotypes are distinguished by distinct combinations of substitutions at amino acid positions 416

(Asp>Glu) and 420 (Lys>Thr). Relative to the wild-type Gc1F protein, the Gc1s is defined by containing the Asp416Glu polymorphism, and the Gc2 contains the Thr420Lys polymorphism. Enzymatic processing of the DBP O-linked carbohydrate to GalNac results in the transformation of the DBP molecules into the macrophage-activating factors (MAF). MAF is a lymphokine that primes macrophages, when required during the host defence responses in infectious and inflammatory diseases and against tumours [12]. Furthermore, MAF can induce macrophage cell death by enhancing caspase activity at sites of infection or inflammation [13].

Several studies have proposed a link between genetic variants of DBP and disease in humans. These include many conditions of suspected autoimmune etiology, such as Graves' disease, diabetes, and osteoporosis [reviewed in 9]. Inflammatory bowel disease (IBD) is a condition characterized by recurrent inflammation of the intestinal mucosa, and is caused by deranged function of mucosal immune system in genetically susceptible subjects [reviewed in 14]. IBD etiology clearly involves a complex interaction of genetic, environmental, and immunomodulatory factors. Two major forms of IBD exist. In Crohn's disease (CD), the entire gastrointestinal tract may be affected, although the most frequent site of inflammation is the ileum, whereas in ulcerative colitis (UC) the inflammation typically affects the colon. In CD pathogenesis, a strong genetic component has been suggested by the concordance of 63.6% in monozygotic twins, but only of 3.6% in dizygotic ones. In UC, the concordance of monozygotic twins is lower (6%), implying that genetic factors may play a somewhat smaller role in this disease [15]. IBD is more prevalent in northern Europe and North America, where less vitamin D is synthesized in the skin upon lower exposure to sunlight [16]. Vitamin D deficiency also frequently occurs in IBD patients, even when the disease itself may be in a state of remission [17].

Genetic variants of the nuclear receptor for vitamin D_3, VDR, the chief executor of vitamin D_3 actions within cell nuclei, have previously been suggested to be associated with inflammatory bowel disease (IBD) [18,19]. Here, we have studied whether two common variants of the other important player in vitamin D homeostasis, DBP, are associated with IBD and its subtypes UC and CD.

Materials and methods

Study subjects

The study population was Swiss, and comprised of 248 healthy subjects and 636 IBD patients, from which 232 were diagnosed to suffer from UC and 404 from CD. The IBD subjects were recruited at the centers participating in the Swiss Inflammatory Bowel Disease Cohort Study (SIBDCS) [20]. For the IBD patients, the diagnosis of UC or CD was confirmed by the study investigators based on clinical presentation, endoscopic findings, and histology. Non-IBD controls were recruited from gastroenterological patients undergoing surveillance colonoscopy. History of colorectal cancer was employed as an exclusion criteria for both IBD patients and non-IBD control subjects.

DNA extraction

Genomic DNAs were extracted from either EDTA-blood or intestinal biopsies using the QIAamp DNA Mini Kit (QIAGEN, Hombrechtikon, Switzerland) or the TRIzol reagent (Invitrogen, Basel, Switzerland), respectively, according to the manufacturer's instructions.

Genotyping of *DBP* single nucleotide polymorphisms

Genotyping of the two *DBP* SNPs was performed using TaqMan allelic discrimination assays. The cycling was performed on an 7900HT Fast Real-Time PCR system (Applied Biosystems, Rotkreuz, Switzerland) by using custom-made TaqMan SNP Genotyping Assays. The TaqMan primer and probe sequences were as follows: The Asp416Glu variant: Left primer, TGGCAGAGCGACTAAAAGCA; right primer, CTTGTTAACCAGCTTTGCCAGTTC; VIC-labelled probe AAATTGCCTGATGCCAC; FAM-labelled probe TTGCCTGAGGCCAC. The Thr420Lys variant: Left primer, CGACTAAAAGCAAAATTGCCTGATG; right primer CTGAGTGCTTGTTAACCAGCTTTG; VIC-labelled probe CACCCACGGAACTG; FAM-labelled probe CACCCAAGGAACTG. All

fluorescent probes had a non-fluorescent quencher at 3'-end. The genomic DNAs were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and diluted to a final concentration of 10 ng/µl. The amplification run conditions were: Once 50°C for 2 min, once 95°C for 10 min, 45 times 95°C for 15 sec and 60°C for 1 min.

Ethical considerations

All subjects provided their written informed consent to be included in the study. Ethical approvals were obtained from the local medical ethical committees of all study sites involved in the Swiss IBD Cohort Study.

Statistical analysis

Statistical analysis was performed using the software packages SPSS 18 (SPSS Inc., Chicago, IL) and GraphPad Prism (GraphPad Software Inc., San Diego, CA). The Chi-square test or Fisher's exact test were used to determine associations between individual SNPs and subject phenotypes. The PHASE software was used to calculate the haplotypes in the *DBP* gene on the basis of a Bayesian interference algorithm [21]. Linkage disequilibrium (LD) was calculated using the r^2 statistics. Calculations were performed using the software package Haploview (www.haploview.com).

Results

Study population

The study population was recruited at centers participating in the Swiss IBD Cohort study (SIBDCS). Detailed demographic data of the entire cohort consisting of 884 subjects is shown in Table 1.

DBP sequence variability

The two *DBP* variants selected for the study are single nucleotide substitutions. The genotype frequencies in all groups were in Hardy-Weinberg equilibrium. The obtained allele and genotype frequencies are given in Table 2.

Genetic variation in the *DBP* gene and IBD

The DBP variant 416 Glu was not found to be significantly associated with the diagnosis of IBD, although there was a tendency for this variant to be more common in the IBD population than in healthy subjects (Tables 2 and 3). The DBP variant 420 Lys, on the other hand, was more common in the healthy population than in the IBD cohort. The minor allele frequency was significantly (p=0.034) lower in the IBD population. The level of significance became much more notable (p=0.006), when the numbers of carriers of the wild-type genotype were compared to the numbers of homozygous SNP carrier in non-IBD and IBD populations. Upon subgrouping the IBD patients, the differences in the frequencies of the rarer 420 Lys variant remained significant for both UC (p=0.022) and CD (p=0.016). Similarly, when adjusted to age and gender, the p values remained significant.

In addition to the two above-mentioned coding *DBP* SNPs, we also genotyped the study population for three *DBP* promoter SNPs, namely -1424, -944, and -39, previously studied in

the context of prostate cancer risk [22]. All the subjects in all subgroups of the current cohort, both IBD and non-IBD, were homozygous for the wild-type allele at these three positions (data not shown).

DBP haplotypes and IBD

All individuals, for whom genotype determination could be performed for both DBP variants under study were included in the haplotype prediction analysis. Thus, 185 non-IBD subjects, 225 UC cases and 393 CD cases were included in this analysis. The predicted frequencies for all four possible *DBP* haplotypes are shown in Table 4. The haplotype *DBP_2* containing the wild-type allele for the position 416 and the rarer allele for the position 420, was predicted to be significantly less frequent in the IBD population than in non-IBD subjects (p=0.0005) and this inverse association with the disease remained significant when either the UC (p=0.0003) or the CD (p=0.013) patients were compared with the non-IBD group separately (Table 5). The linkage equilibria between the variants at positions 416 and 420 of DBP were moderate (non-IBD controls r^2=0.61; IBD cases r^2=0.78).

Discussion

The etiology of IBD is complex, and a wide range of factors, both genetic and environmental, are believed to play roles in IBD pathophysiology. Variants in more than 70 genes have been identified that may potentially be associated with IBD [23,24]. Many of these encode genes that modulate immune responses and antimicrobial defence in the intestine, and are thus involved in maintaining the integrity of the intestinal wall epithelium [14,23]. Vitamin D metabolites have anti-inflammatory functions, and participate in the maintenance of tight junctions between intestinal epithelial cells [2]. While the other major player of vitamin D homeostasis, the vitamin D receptor VDR, has previously been genetically linked to IBD [18,19], we show here that a common genetic variant of DBP, the main transporter of vitamin D to its target tissues in the plasma, is significantly associated with IBD. This association is significant in both our UC and CD cohorts. The rarer Lys allele at position 420 appears to have a protective role against IBD, as it is more common in healthy subjects. The other DBP variant tested 416 Glu did not alone show a significant association with IBD, although it exhibited a tendency to be more frequent in IBD cases than control subjects, potentially thus acting as a factor contributing to the pathogenesis. The haplotype *DBP_2*, consisting of variants 416 Asp and 420 Lys, was significantly more frequent in the non-IBD population, more notably in comparison with the UC (OR 4.390).

Low 25-hydroxyvitamin D_3 levels are associated with insulin resistance and metabolic syndrome, and genetic variation in codon positions 416 and 420 has previously been proposed to be associated with diabetes [25,26], although this association remains somewhat controversial [27,28]. Furthermore, the DBP Lys allele at codon 420 has been associated with Graves' disease in a Polish population [29], although in this case the lysine was in fact more common in the disease group. This points to potentially differing roles of

DBP in the etiologies of IBD and Graves' disease, even if both represent autoimmune disorders.

The exact functional consequences of the amino acid exchanges at positions 416 and 420 of the DBP protein remain somewhat unclear. This region of the protein is located adjacent to the actin-binding domain of DBP (amino acids 350-403), but is more distant from the amino-terminal vitamin D-binding domain [30]. In the DBP form carrying the lysine in position 420, a major site for O-linked trisaccharide glycosylation is removed [31]. It will be of great interest to verify whether the efficiency of vitamin D transport by DBP is affected by this amino acid substitution, and in what manner. Given its inverse association with IBD reported here, it is perhaps paradoxical that the Gc2 isoform of DBP carrying the lysine in codon position 420 has been associated with lower plasma concentrations of vitamin D_3 metabolites than observed for the carriers of the other Gc alleles [11]. It has also been shown that the Gc2 isoform has reduced affinity to vitamin D metabolites [32]. We do not at this stage understand the exact mechanism by which a DBP isoform that exhibits lower affinity to vitamin D exerts a protective effect against IBD, as suggested by our current findings. It is conceivable that the reduced affinity could result in enhanced release of vitamin D from DBP to the intestinal target tissue that is susceptible to inflammation.

Patients suffering from IBD are frequently on long-term treatment with vitamin D_3, in addition to calcium, as a prophylaxis against osteoporosis and osteopenia [33]. It will be of interest to further explore whether the IBD-associated DBP variants are associated with altered clinical response to vitamin D_3 supplementation. In conclusion, we have for the first time shown an association between a chief component of vitamin D metabolism, DBP, and diagnosis of IBD, further supporting the importance of vitamin D homeostasis in this chronic inflammatory disease.

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Footnotes

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